Covalent Modification and Active Site-Directed Inactivation of a Low Molecular Weight Phosphotyrosyl Protein Phosphatase†

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ABSTRACT: Covalent modification experiments were conducted in order to identify active site residues of the 18-kDa cytoplasmic phosphotyrosyl protein phosphatases. The enzyme was inactivated by diethyl pyrocarbonate, phenylglyoxal, cyclohexanedione, iodoacetate, iodoacetamide, phenylarsine oxide, and certain epoxides in a manner consistent with the modification of active site residues. Phenylglyoxal and cyclohexanedione both bind to the active site in a rapid preequilibrium process and thus act as active site-directed inhibitors. The pH dependencies of the inactivation by iodoacetate and by iodoacetamide were examined in detail and compared with rate data for the alkylation of glutathione as a model compound. The enzyme inactivation data permitted the determination of pK_a values of two reactive cysteines at or near the active site. Although phosphomycin is simply a competitive inhibitor of the enzyme, it was found that 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and (*R*)- and (*S*)-benzylglycidol act as irreversible covalent inactivators, consistent with the importance of a hydrophobic moiety on the substrate in controlling substrate specificity. EPNP exhibits characteristics of an active site-directed inactivator, with a preequilibrium binding constant somewhat smaller than that of phosphate ion. The pH dependencies of inactivation of EPNP and (*S*)-benzylglycidol are identical to that observed for iodoacetamide and similar to that for iodoacetate, suggesting that they modify similar groups. Sequencing of the tryptic digests of the EPNP-labeled enzyme indicates that Cys-62 and Cys-145 are labeled. Phenylarsine oxide acts as a very slow, tight-binding inhibitor of the enzyme. The results are interpreted in terms of an active site model that incorporates a histidine–cysteine ion pair, similar to that present in papain.

The reversible phosphorylation of proteins catalyzed by protein kinases and protein phosphatases is now recognized to be a major process for regulating cellular functions. This phosphorylation occurs not only on serine and threonine but also on tyrosine residues (Edelman et al., 1987; Cohen, 1989; Fischer et al., 1991). Both soluble and particulate forms of protein tyrosine phosphatases are now being identified. The membrane-associated, receptor-linked protein tyrosine phosphatases may function to provide a new intracellular signaling pathway mediated by protein dephosphorylation, thus controlling cell proliferation and cell–cell communication (Shenolikar & Nairn, 1991). In contrast, the role of cytoplasmic protein tyrosine phosphatases remains largely unresolved.

Low molecular weight acid phosphatases [EC 3.1.3.2, orthophosphoric monoester phosphohydrolases (acid optimum)] are found in the cytosol of most cells (DeAranjo et al., 1976; Harris, 1980; Chen & Chen, 1988; Dissing & Svensmark, 1990; Zhang, 1990). These enzymes are active phosphotyrosyl protein phosphatases and have been shown to readily hydrolyze substrates that include phosphotyrosyl (but not phosphoseryl) casein, IgG, red cell band 3, angiotensin, tyrosine kinase P^{40}, and epidermal growth factor (Zhang & Van Etten, 1990, and references cited therein; Boivin et al., 1987). Along with other structurally distinct members of the phosphotyrosyl protein phosphatase family, these enzymes may be expected to play a key role in cellular regulatory processes, for example in the control of signal transduction involving the tyrosine kinase activities of receptors for peptide hormones and growth factors or affecting the activity of glycolytic enzymes (Harrison et al., 1991).

Some of the kinetic and mechanistic features of the hydrolysis of phosphate monoesters catalyzed by bovine heart phosphotyrosyl protein phosphatase have been extensively characterized. The existence of a covalent phosphoenzyme intermediate on the pathway of the phosphatase-catalyzed hydrolysis of phosphate monoesters has been implicated through the use of a variety of methods including pre-steady-state, steady-state, and ^{18}O-exchange experiments, and the rate-determining step has been identified as the breakdown of this intermediate (Zhang & Van Etten, 1991a). In addition, a structure–activity correlation of leaving-group effects on the phosphorylation of the enzyme indicated a strong electrophilic participation in the transition state for this catalytic event, while a proton inventory study implicated the involvement of a solvent-derived proton in the transition state (Zhang & Van Etten, 1991b).

However, little is known about the chemical nature of the enzyme active site. Previous studies on the low molecular weight phosphotyrosyl protein phosphatase established the critical requirement of free sulfhydryls for enzyme activity (Chaimovich & Nome, 1970; Lawrence & Van Etten, 1981; Taga & Van Etten, 1982; Laidler et al., 1982; Waheed et al., 1988; Camici et al., 1989). At the same time, a loss of enzyme activity also occurs upon photoinactivation in the presence of Rose Bengal dye (Lawrence & Van Etten, 1981) and upon incubation with diethyl pyrocarbonate (Taga & Van Etten, 1982; Dayton, 1987; Waheed et al., 1988). The latter experiment in particular would seem to suggest the importance of one or more histidine residues.

Since most of the earlier experiments were done at one particular pH and inactivator concentration, we have undertaken a more systematic approach that involves the study of the pH dependence, the concentration dependence, the D_2O solvent isotope effect, the effects of alternate inactivation

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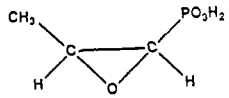
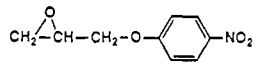
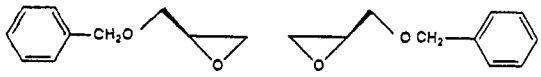


Phosphomycin



1,2-Epoxy-3-(*p*-Nitrophenoxy)-Propane



(R)-(-) and (S)-(+)-2-(Benzyloxymethyl)-Oxirane

FIGURE 1: Structures of phosphomycin, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and (*R*)-(−)- and (*S*)-(+)-2-((benzyloxy)methyl)oxirane (benzylglycidol).

reagents, and the effects of competitive inhibitors upon enzyme inactivation. It was also necessary to reexamine several earlier experiments because they were done in cacodylate buffer, which is now recognized to be a competitive inhibitor of the bovine liver enzyme (Dayton, 1987).

Another important objective of the present study was to attempt to discover a possible affinity label for phosphatases that have reactive, active site nucleophiles. Affinity labeling represents a sophisticated way of selectively modifying active site residues, by utilizing the capacity of a protein to form complexes as it carries out its biological function (Baker, 1967; Plapp, 1982; Colman, 1983). Enzyme inactivation using active site-directed inactivators often provides important information regarding the nature and location of the residues being modified. Unfortunately, there are no reports of such work on phosphotyrosyl protein phosphatases. On the basis of earlier substrate specificity studies (Zhang, 1990; Zhang & Van Etten, 1990) and information obtained from the present chemical modification reactions, we have selected and tested a group of potential active site-directed inactivators. Several reagents are identified that may have broad applicability in studies of phosphotyrosyl protein phosphatases.

Experimental Procedures

Materials. Except for the experiments involving reaction with phenylarsine oxide (PAO),[1] the low molecular weight phosphotyrosyl protein phosphatase (BHPTP) was purified to homogeneity from bovine heart (Zhang & Van Etten, 1990). Bovine hearts were obtained from a local slaughterhouse. The PAO experiments were done using recombinant enzyme (Wo et al., 1992). *p*-Nitrophenyl phosphate (*p*NPP), phenylglyoxal, 1,2-cyclohexanedione, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione, diethyl pyrocarbonate (DEP), iodoacetate, iodoacetamide, PAO, and phosphomycin were from Sigma. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane (EPNP) was from Kodak, while D_2O (99.9%), (*R*)-(−)- and (*S*)-(+)-2-((benzyloxy)methyl)oxirane (also called (*R*)- and (*S*)-benzylglycidol) were from Aldrich. The structures of several of these potential affinity labels are shown in Figure 1. All other reagents were analytical grade and were used without further purification. The buffers were prepared using twice-deionized and distilled water.

Covalent Modification Reactions. The modification of arginine residues of BHPTP was conducted in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M, pH 7.0, buffer at 27 °C. For the phenylglyoxal concentration dependence study, the inactivation reaction was started by adding a few microliters of a concentrated phenylglyoxal stock solution (prepared in absolute ethanol just before the experiment) to the inactivation mixture which contained 8 μg of homogeneous BHPTP. The total reaction volume was 200 μL, and the in situ concentrations of reagent were 0.4, 0.5, 0.6, 1.0, 1.5, and 2.0 mM. A control sample was prepared containing only 8 μg of enzyme and buffer, using an aliquot of absolute ethanol in place of reagent. At intervals of time, 10-μL aliquots were taken and the enzyme activity was assayed for 3 min at 37 °C in pH 5 assay buffer containing 10 mM *p*NPP. The percentage of residual activity of the modified enzyme sample was calculated relative to the control sample, and the pseudo-first-order rate constant for the inactivation was determined by the linear regression program PLOT (Mega, 1989). The effect of an added competitive inhibitor was assessed in the same manner, except that two samples were prepared, one with phenylglyoxal alone and the other with phenylglyoxal plus competitive inhibitor. The inactivation of BHPTP using 1,2-cyclohexanedione was performed in the same way as described for the phenylglyoxal reactions.

The free sulfhydryl content of the enzyme after the modification of arginine was determined using DTNB (Laidler et al., 1982). Briefly, 32 μg of homogeneous BHPTP was incubated with 0.5 mM phenylglyoxal in the presence and absence of 10 mM phosphate. The reaction was followed until less than 5% of enzyme activity remained. Then, aliquots of 10 mM DTNB were added to the enzyme samples and to the control. The number of free sulfhydryl groups was determined by measuring the absorbance at 412 nm, using an extinction coefficient of 13 600 M^{-1} cm^{-1}.

Modification reactions of histidine residues with DEP, as well as studies of the effects of phosphate and DEP concentrations on the reactions, were carried out in the same manner as described above. The actual concentrations of DEP in the solutions were determined from the stoichiometry of reaction with imidazole at pH 6.0, in 0.1 M 3,3-dimethylglutarate buffer, using the increase in the absorbance at 230 nm of $\Delta\epsilon$ = 3000 M^{-1} cm^{-1} (Melchior & Fahrney, 1970; Miles, 1977). Fresh stock solutions of DEP were prepared in absolute ethanol immediately before use. Buffers used were as follows: from pH 4 to 5.6, 100 mM acetate; from pH 6.0 to 7.0, 50 mM 3,3-dimethylglutarate; and at pH 7.5, 100 mM Tris. In all the buffer systems, 1 mM EDTA was included and the ionic strength was kept at 0.15 M, with adjustments made as necessary by the addition of NaCl. Because DEP undergoes hydrolytic decomposition at a rate that may be comparable to or even greater than the rate at which it reacts with enzyme, the rate constant for enzyme inactivation was obtained by fitting the activity versus time data to eq 1. In this expression,

$$[E] = [E]_0 \exp[(k_2[DEP]_0/k_{sp})(e^{-k_{sp}t} - 1)] \quad (1)$$

[E] is the remaining enzyme concentration, $[E_0]$ is the initial enzyme concentration, k_2 is the rate constant for enzyme inactivation, and k_{sp} is the rate constant for spontaneous decomposition of DEP. Two samples were prepared that contained 10 μg of BHPTP, 30 mM NH_2OH in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M, pH 7.0, buffer. One of them also contained 10 mM phosphate, while the other did not. They were incubated at 25 °C until all the enzyme

[1] Abbreviations: PAO, phenylarsine oxide; BHPTP, bovine heart phosphotyrosyl protein phosphatase; *p*NPP, *p*-nitrophenyl phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DEP, diethyl pyrocarbonate (ethoxyformic anhydride); GSH, reduced glutathione; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; TPCK, tosylphenylalanine chloromethyl ketone; DTT, dithiothreitol; TFA, trifluoroacetic acid.

activity was effectively zero. Then, the samples were analyzed by SDS–PAGE (Laemmli, 1970).

The experimental procedures for the modification of BHPTP using iodoacetate and iodoacetamide utilized the following buffers: from pH 5 to 5.6, 100 mM acetate; from pH 6.0 to 7.2, 50 mM 3,3-dimethylglutarate; from pH 7.5 to 8.7, 100 mM Tris; and from pH 9.0 to 10.0, 100 mM glycine. In all the buffer systems, 1 mM EDTA was included and the ionic strength was kept at 0.15 M (adjusted by additions of NaCl). The D_2O solvent isotope effects on the alkylation of BHPTP by iodoacetate and iodoacetamide were measured at pL 5.0 (that is, within the pL-independent region of the modification, where L = H or D) in 100 mM acetate, 1 mM EDTA, I = 0.15 M buffer at 37 °C. The alkylation of reduced glutathione (GSH) was followed in the same manner as for the enzyme-modification reaction. Briefly, 0.5 mM GSH was incubated with 5 mM iodoacetate or iodoacetamide in buffer solutions. At various time intervals, 100-μL aliquots were withdrawn and added immediately to 0.9 mL of a solution of 1 mM DTNB in 0.1 M 3,3-dimethylglutarate, pH 7, buffer, containing 1 mM EDTA. The free sulfhydryl content was quantitated by measuring the absorbance at 412 nm (ϵ = 13 600 M^{-1} cm^{-1}).

The concentration of EPNP was determined spectroscopically since EPNP has an absorption maximum at 315 nm with an extinction coefficient of 11 000 M^{-1} cm^{-1} in water (Chen & Tang, 1972). Buffer systems used were as follows: pH 3.5, 100 mM 3,3-dimethylglutarate; pH 4.0 and 5.0, 100 mM acetate; pH 6.0, 6.6, 7.0, and 7.1, 50 mM 3,3-dimethylglutarate; pH 7.5, 8.0, 8.3, and 8.5, 100 mM Tris; pH 9.0 and 9.5, 100 mM glycine. Each buffer also contained 1 mM EDTA and the ionic strength was 0.15 M, adjusted by addition of NaCl. The reaction temperature was 27 °C. The BHPTP inactivations by (*R*)- and (*S*)-benzylglycidol were conducted in the same manner as that described for EPNP. The buffer systems were also the same. All experiments were done at 25 °C.

Kinetic data were fitted to the indicated expressions using the nonlinear multiple regression program MULTI (Yamaoka et al., 1981).

Active Site Labeling and Proteolysis. The labeling of BHPTP by EPNP was started by adding BHPTP (83–320 μg, 113 units/mg) to 2.4 mM EPNP dissolved in 200 mM Tris, pH 8.1, buffer at room temperature. The total volume of the reaction mixture was 120 μL, and the final EPNP concentration was 1.2 mM. The reaction was allowed to proceed for 3.5 h, yielding an enzyme sample with less than 10% of remaining activity. The volume was quickly (15–30 min) reduced to 40 μL using a Speed-Vac concentrator, and then 60 μL of 4 M urea (Sequanal grade) in the same pH 8.1 Tris buffer was added to the concentrated modified enzyme sample. Immediately, 10 μg of trypsin and 0.1 mM TPCK were introduced to start the trypsin cleavage reaction. The final urea concentration was 2 M. The cleavage reaction was conducted at 37 °C for 2.5 h. Then, the tryptic digest mixture was reduced with 5 mM DTT for 2 h, and 10 mM iodoacetate was added following the reduction to completely carboxymethylate the unlabeled Cys residues. The carboxymethylation reaction was performed in the dark for 20 min. A control sample, from which BHPTP was omitted, was prepared and handled exactly in the same manner. It was also established that, under these conditions, EPNP does not react with the sulfhydryl group in GSH, as confirmed by using the same assay procedure as developed for the alkylation of GSH by iodoacetate. Thus, the possibility of nonspecific reactions of cysteine residues is unlikely.

Table I: Effects of Competitive Inhibitors on the Inactivation of BHPTP by Phenylglyoxal[a]

[phenylglyoxal] = 0.5 mM		[phenylglyoxal] = 0.7 mM	
[phosphate] (mM)	k_1 (min^{-1})	[arsenate] (mM)	k_1 (min^{-1})
0	0.0266	0	0.0463
10	0.0240	5	0.0238

[a] The experiments were performed at pH 7.0 and 27 °C in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M buffer. k_1 is the pseudo-first-order rate constant of inactivation.

The entire carboxymethylated tryptic digest was directly analyzed by reversed-phase HPLC without further sample manipulation. An IBM LC/9533 HPLC system equipped with a 4.6 × 250 mm Synchropak RP-P C18 column was used to separate the products in the tryptic digest. Solvent A was 0.1% TFA in water, and solvent C was 0.1% TFA in acetonitrile. The flow rate was set at 1.0 mL/min, with a linear gradient of 1% increment in solvent C per min. The fractions (1 mL) were monitored at two wavelengths, 210 nm for peptides and 315 nm for EPNP labels. The peptide sequencing was done on either an Applied Biosystems 473A pulsed-liquid protein sequenator or an Applied Biosystems 470A gas-phase sequenator.

Inactivation of the Enzyme by Phenylarsine Oxide. Inactivation of the enzyme by phenylarsine oxide (PAO) was carried out in a 20 mM 3,3-dimethylglutarate buffer, pH 7.0, with 1 mM EDTA added. The enzyme concentration was 4 μM. PAO was initially dissolved in ethanol to give a 5 mM stock solution, and diluted portions were added to the buffered enzyme solution. Aliquots (10 μL) were taken at various times and assayed for activity. For larger scale preparations of inactivated enzyme, 50–100 μM PAO was incubated overnight with 0.1–0.3 mg of recombinant enzyme. The resulting inactive complex was isolated and freed from excess PAO by G-25 Sephadex chromatography.

Results

Modification of Arginine. BHPTP was found to be effectively inactivated by phenylglyoxal at pH 7.0 and 27 °C. Although phenylglyoxal is fairly selective for arginine, especially at lower pH values such as pH 7.0, the possibility that the inactivation caused by phenylglyoxal was due to a side reaction with cysteine residues was examined by determining the free sulfhydryl content of the protein after the modification. The protein is known to contain eight free cysteines (Wo et al., 1992). The results showed that the phenylglyoxal modification did not involve a cysteine residue, since in every case, the number of cysteine residues per enzyme molecule remained the same before and after the phenylglyoxal treatment, in both the control and the samples containing phenylglyoxal, with and without phosphate. The possibility that the reaction involved the amino terminus is ruled out, since the N-terminal residue of the bovine heart enzyme that is isolated from tissue is acetylated (Wo et al., 1992).

The effects of competitive inhibitors on the kinetics of inactivation are summarized in Table I. It is likely that the phenylglyoxal modification is directed toward the enzyme active site, since both phosphate and arsenate provide protection against the inactivation. Both ions are competitive inhibitors of the bovine enzyme, although arsenate is a stronger inhibitor (Zhang & Van Etten, 1990). Thus, the involvement of one or more essential arginine residues in the action of low molecular weight phosphotyrosyl protein phosphatase is implicated.

The results of the concentration-dependent inactivation of the enzyme by phenylglyoxal at pH 7.0, 27 °C, are summa-

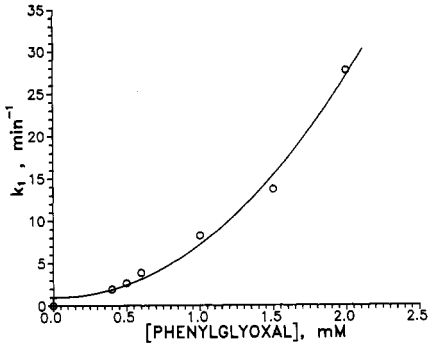


FIGURE 2: Concentration dependence for the inactivation of enzyme by phenylglyoxal at pH 7.0 and 27 °C. The line was obtained by direct fit of the experimental data to the expression $k_{exp} = k'[I]^n + C$, and it gave a kinetic order $n = 2.07 \pm 0.28$ with respect to phenylglyoxal.

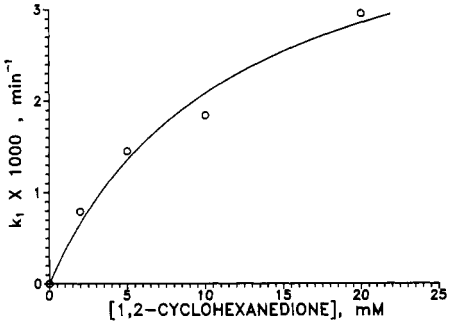


FIGURE 3: Concentration dependence of the inactivation of the enzyme by 1,2-cyclohexanedione. The experiment was conducted at pH 7.0 and 25 °C. The line was obtained by direct fit of the experimental data to the expression $k_{exp} = k'[R]/([R] + K_i)$, giving $K_i = 11.7 \pm 4.0$ mM.

rized in Figure 2. By fitting the experimental rate constants k_{exp} to the expression $k_{exp} = k'[I]^n + C$, a kinetic order $n = 2.07 \pm 0.28$ was obtained with respect to phenylglyoxal. Earlier studies with model compounds found that the stoichiometry of the reaction of phenylglyoxal with arginine requires 2 mol of phenylglyoxal for each molar equivalent of arginine residue (Takahashi, 1968; Lange et al., 1974; Cheng & Nowak, 1989). However, examples are also known where a 1:1 stoichiometry was found, depending on the particular local conformation of the enzyme active site (Borders & Riordan, 1975; Gildensoph & Briskin, 1989; McKee & Nimmo, 1989). In any event, it appears that at least one arginine is essential for BHPTP activity.

The modification of arginine using another commonly used arginine-selective reagent, 1,2-cyclohexanedione, was also examined. The concentration dependence for inactivation by 1,2-cyclohexanedione at 25 °C, pH 7.0, displays saturation kinetics with $K_i = 11.7 \pm 4.0$ mM (Figure 3). That is, the enzyme forms a complex with cyclohexanedione prior to the covalent modification event. At pH 7.0 and 25 °C, the rate constants for inactivation by 10 mM 1,2-cyclohexanedione in the absence and presence of 10 mM inorganic phosphate were 1.84×10^{-3} and 6.77×10^{-4} min^{-1}, respectively. At pH 8.3 and 25 °C, the rate constants of inactivation by 10 mM 1,2-cyclohexanedione in the absence and presence of 10 mM phosphate were 2.39×10^{-2} and 2.04×10^{-2} min^{-1}, respectively. The fact that inorganic phosphate provides less protection against 1,2-cyclohexanedione inactivation at pH 8.3 than at pH 7.0 is consistent with the fact that phosphate ion is a much weaker competitive inhibitor at pH values above 8 (Zhang, 1990).

Histidine Modification. The rate constants for inactivation

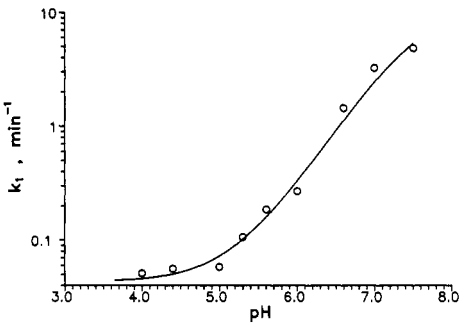


FIGURE 4: pH dependence of enzyme inactivation by diethyl pyrocarbonate. Experiments were performed at pH 6.0 and 37 °C. The initial DEP concentration was 10 mM.

of BHPTP were 0, 0.140, 0.246, 0.430, 0.577, 0.713, and 0.927 min^{-1} at initial DEP concentrations of 0, 3, 7, 12, 15, 18, and 25 mM, respectively. These data were fitted to the expression $k_{exp} = k'[I]^n + C$ and gave an n value of 0.997 ± 0.078. The presence of inorganic phosphate afforded protection against the inactivation of BHPTP by DEP. At pH 6.0 and 37 °C, the rate of inactivation of BHPTP by 10 mM DEP in the absence of phosphate was 0.383 min^{-1}, while in the presence of 10 mM phosphate, the rate constant was 0.096 min^{-1}. The DEP concentration dependence of the inactivation is consistent with the conclusion that only one histidine residue was modified by DEP at pH 6.0, while the protection afforded by phosphate suggests that the histidine residue is located at or near the enzyme active site. However, the addition of 100 mM hydroxylamine did not restore any enzyme activity following DEP modification.

The pH dependence of DEP modification at a 10 mM DEP concentration is shown in Figure 4. The result indicates that the residue being modified by DEP has an apparent pK_a above 7. pK_a values were estimated by fitting the data to eq 2 using a nonlinear least-squares fitting program (Yamaoka et al., 1981).

$$k_1 = \frac{k_a[E_o] + k_b[E_o](K_a/[H^+])}{1 + (K_a/[H^+])} \quad (2)$$

Alkylation of Glutathione with Iodoacetate and Iodoacetamide. Reduced glutathione (GSH) was chosen as a model compound for a comparison of the effects on the alkylation of sulfhydryls by iodoacetate and iodoacetamide. Results from these model studies proved to be useful in interpreting the results that were obtained with the enzyme system. At iodoacetamide concentrations of 3, 5, 6, 8, and 10 mM, the alkylation of GSH by iodoacetamide at pH 7.0 and 37 °C gave rate constants of 0.100, 0.152, 0.255, 0.270, and 0.380 min^{-1}, respectively. By fitting the data to the expression $k_{exp} = k'[I]^n + C$, a kinetic order n of 1.09 ± 0.24 was obtained. The alkylation involved 1 mol of GSH reacting with 1 mol of iodoacetamide. Furthermore, alkylation of GSH at pH 9.0, 37 °C, in H_2O and D_2O did not reveal significant solvent isotope effects for reaction with either iodoacetate or iodoacetamide (data not shown).

The pH dependencies of the alkylation of GSH by 5 mM iodoacetate and by 5 mM iodoacetamide at 37 °C are summarized in Figure 5. A pK_a of 8.62 ± 0.06 was found from the iodoacetate reaction, while a pK_a of 8.79 ± 0.08 was found from the iodoacetamide reaction. These values are in good agreement with reported pK_a values for GSH of between 8.64 and 8.75 for the –SH and between 9.52 and 9.65 for the α-ammonium group (Gerwin, 1967; Lindley, 1962; Martin & Edsall, 1958). Note that, at each pH, the rate of alkylation with iodoacetamide is approximately 3.5-fold faster than that

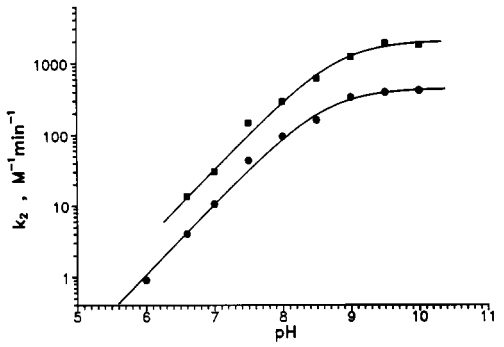


FIGURE 5: pH dependence of the alkylation of GSH by iodoacetate and iodoacetamide at 37 °C. Symbols: (■) 5 mM iodoacetamide; (●) 5 mM iodoacetate.

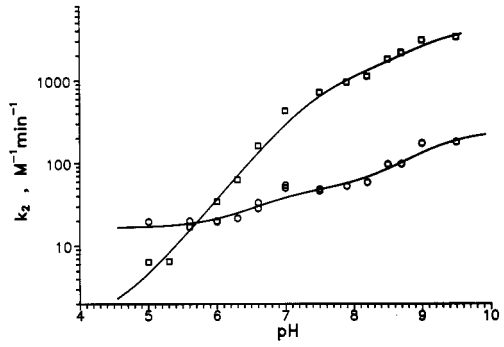


FIGURE 6: pH dependence for the inactivation of BHPTP by iodoacetate (○) and iodoacetamide (□) at 37 °C.

with iodoacetate. This is consistent with the fact that the carboxamido group is more electron withdrawing than the corresponding carboxylate group, thus making iodoacetamide more susceptible to attack by the sulfur nucleophile.

Modification of BHPTP with Iodoacetate. At pH 6.0 and 37 °C, the first-order rate constants for inactivation by 5 mM iodoacetate in the presence of 0, 10, and 30 mM inorganic phosphate were 0.086, 0.016, and 0.0097 min^{-1}, respectively. Table II summarizes the concentration dependence for modification by iodoacetate at that pH and temperature and in the same buffer. The kinetic order was calculated from the expression $k_{exp} = k'[I]^n + C$ and an n value of 1.08 ± 0.07 was obtained. The concentration dependence of the cysteine modification by iodoacetate was also studied at pH 8.5 and 37 °C, in 100 mM Tris, 1 mM EDTA, I = 0.15 M buffer. At that pH, the kinetic order n was 1.00 ± 0.02 with respect to iodoacetate.

The rate constants of the iodoacetate modification reaction were measured as a function of pH using a 5 mM reagent concentration at 37 °C. A logarithmic plot of the second-order rate constants for inactivation versus the pH is shown in Figure 6. Unlike the model reaction with GSH, the log (k_2) versus pH curve for modification of the enzyme did not define a simple ionization. Instead, ionic interactions appeared to give rise to some degree of negative cooperativity which affected the rate of the modification reaction. The pK_a values of the ionizable groups could be determined by directly fitting the experimental second-order rate constants k_2 to eq 3. The data

$$k_2 = \frac{k_l[E_o]\frac{[H^+]}{K_1} + k_m[E_o] + k_u[E_o]\frac{K_2}{[H^+]}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (3)$$

were fitted using separate terms for the rate components corresponding to EH_2, EH, and E (k_l, k_m, and k_u, respectively) and assuming only two ionizable groups[2] having acid ionization constants of K_1 and K_2. The pH dependence of the enzyme inactivation by iodoacetate suggested that two cysteine residues having pK_a values of 6.75 ± 0.34 and 9.12 ± 0.30 were being modified (Figure 6). As a rule, ionized thiolate anions are much more reactive than the SH form in reactions with alkyl halides. Thus, the alkylation of free sulfhydryl groups as a function of pH should give a normal titration curve, reflecting the pK_a of the SH group. However, Figure 6 shows that the iodoacetate-promoted inactivation still proceeds at a significant rate at pH 5, even though at that pH cysteine residues are expected to be in their unionized SH form.

Modification of BHPTP with Iodoacetamide. At pH 6.0 and 37 °C, the rate constants for inactivation of BHPTP by 2 mM iodoacetate in the presence of 0 and 10 mM inorganic phosphate were 0.0697 and 0.0103 min^{-1}, respectively. At pH 7.75 and 37 °C, the rate constants for inactivation by 0.5 mM iodoacetamide in the presence of 0 and 10 mM phosphate were 0.562 and 0.391 min^{-1}, respectively. At pH 9.0 and 37 °C, the rate constants for inactivation by 0.4 mM iodoacetamide in the presence of 0 and 10 mM phosphate were 1.31 and 1.25 min^{-1}, respectively. Again, the extent of protection provided by phosphate decreases as the pH increases, due to the decreasing affinity of the enzyme for phosphate (Zhang, 1990). Nonetheless, phosphate clearly provides protection against the

Table II: Rate Constants for Inactivation of BHPTP as a Function of Iodoacetate Concentration[a]

[iodoacetate] (mM)	buffer	k_1 (min^{-1})
0.5	a	0.0110
1.0	a	0.0195
2.0	a	0.0358
4.0	a	0.0647
5.0	a	0.0891
6.0	a	0.101
8.0	a	0.143
10.0	a	0.164
12.0	a	0.221
0.50	b	0.0641
1.0	b	0.125
2.0	b	0.242
4.0	b	0.491

[a] Experiments were conducted at 37 °C. Buffer a: pH 6.0, 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M. Buffer b: pH 8.5, 100 mM Tris, 1 mM EDTA, I = 0.15 M.

Table III: Rate Constants for Inactivation of BHPTP as a Function of pH and Iodoacetamide Concentration

pH	[iodoacetamide] (mM)	k_1 (min^{-1})
6.0	1.0	0.0343
6.0	2.0	0.0697
6.0	5.0	0.174
7.5	1.0	0.718
7.5	5.0	3.09
7.8	0.5	0.562
7.8	1.0	1.16
9.0	0.1	0.425
9.0	0.2	0.768
9.0	0.4	1.31
9.0	0.5	1.63
9.0	0.8	2.65
9.0	1.0	3.07

[a] The reaction temperature was 37 °C. The buffers that were used are specified in the Experimental Procedures section.

[2] If only one ionizable group was assumed, there were large systematic deviations for these data as well as for the iodoacetamide and EPNP rate data. No such systematic deviations were seen if two ionizable groups were assumed.

inactivation by iodoacetamide. The concentration dependence of the iodoacetamide modification reaction at pH 6.0, 7.5, 7.8, and 9.0 is summarized in Table III. The kinetic order n values with respect to iodoacetamide at pH 6.0, 7.5, 7.8, and 9.0 were determined to be 1.00 ± 0.01, 0.91 ± 0.10, 1.04 ± 0.05, and 0.95 ± 0.11, respectively. Results obtained for the pH dependence of the inactivation of BHPTP by iodoacetamide, using a 1 mM reagent concentration at 37 °C, are also shown in Figure 6. The data were analyzed as described for the reaction with iodoacetate. Again, two ionizable groups were indicated,[2] this time with pK_a values of 7.52 ± 0.16 and 9.05 ± 0.40.

From Figure 6, several differences are apparent between the iodoacetate- and iodoacetamide-promoted enzyme inactivation. First, although both iodoacetate and iodoacetamide react with BHPTP at low pH values, iodoacetate reacts about 3-fold *faster* than iodoacetamide below pH 5.3. In contrast, in the model studies employing GSH, the rate of alkylation by iodoacetate is about 3.5-fold *slower* than that with iodoacetamide at each pH. Second, at intermediate pH values, the pH dependence of the inactivation of BHPTP by iodoacetamide is distinctly different from that for the inactivation by iodoacetate. In addition, iodoacetamide is more reactive (about 2-fold at pH 6 to about 15-fold at pH 7.5) than iodoacetate. Third, above pH 7.5, iodoacetamide is about 20-fold more reactive than iodoacetate, but they both exhibit similar pH dependencies as the pH increases.

D_2O Solvent Isotope Effect on the Cysteine Modification Reactions. The effects of D_2O on the inactivation of enzyme by iodoacetate and by iodoacetamide were studied at pL = 5.0. For 5 mM iodoacetate, the first-order rate constant for the inactivation reaction was 0.0961 min^{-1} in H_2O, while in D_2O it was 0.127 min^{-1}. Thus, a moderate inverse solvent isotope effect k^{H_2O}/k^{D_2O} = 0.757 was observed for the reaction between BHPTP and ICH_2COO^-. For 5 mM iodoacetamide, the first-order rate constant for inactivation was 0.0178 min^{-1} in H_2O, while in D_2O the first-order rate constant for inactivation was 0.0173 min^{-1}. Thus, k^{H_2O}/k^{D_2O} = 1.03, showing that there is no solvent isotope effect for the reaction between BHPTP and ICH_2CONH_2.

Modification by Potential Affinity Reagents. Attempts to inactivate the enzyme using phosphomycin were conducted at 27 °C in 0.1 M acetate, 1 mM EDTA, pH 5.0, I = 0.15 M buffer. In the presence of 5 mM phosphomycin, the enzyme did not exhibit a time-dependent loss of activity even after 12 h. At the same pH, even using 100 mM phosphomycin, no time-dependent inactivation occurred after 1 h of incubation at 37 °C. Similar observations were made at pH 7.0, in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M buffer and at pH 7.5, in 0.1 M Tris, 1 mM EDTA, I = 0.15 M buffer using 10 mM phosphomycin, at 27 °C. Thus, phosphomycin, under the conditions studied here, does not covalently modify the enzyme. Instead, it acts as a simple reversible inhibitor. Competitive inhibition constants could be estimated for phosphomycin at 27 °C and pH 5.0, 7.0, and 7.5. They were 4.6, 2.4, and 2.8 mM, respectively. At 37 °C and pH 5, K_I was 15 mM.

In contrast, the epoxide EPNP readily inactivated the enzyme in an irreversible manner. The pH dependence of the inactivation of BHPTP by EPNP is shown in Figure 7. Using a nonlinear least-squares analysis (Yamaoka et al., 1981), the data were fitted to eq 3, where k_l, k_m, and k_u represent reaction rate constants corresponding to reaction of EH_2, EH, and E. In this way, the pH dependence of the inactivation by EPNP was fitted assuming only two ionizable groups, and the pK_a values of these groups were found to be 7.4 ± 0.3 and 9.2 ± 1.6, respectively. Interestingly, these two pK_a values are exactly the same as those determined in the iodoacetamide inactivation experiments. Since EPNP is not charged, the reaction between EPNP and BHPTP does not show the perturbed ionization that is observed in the case of reaction with iodoacetate.

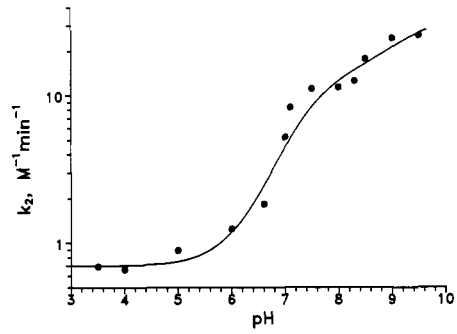


FIGURE 7: pH dependence of the inactivation of BHPTP by EPNP. The experiments were done at 27 °C, and the buffers used were listed in Experimental Procedures. The EPNP concentration was 0.704 mM.

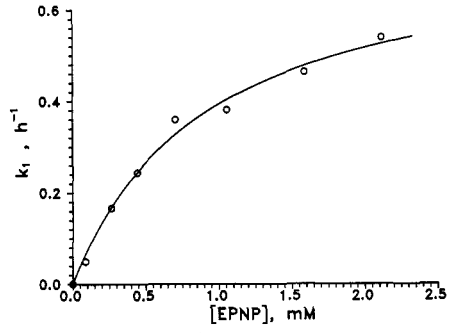


FIGURE 8: Saturation kinetics of the modification of BHPTP by EPNP. The experiment was performed by pH 7.1 and 27 °C in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M buffer.

The concentration dependence of the inactivation of BHPTP by EPNP showed that the modification by EPNP exhibited saturation kinetics (Figure 8). Thus, EPNP first binds to the enzyme active site and then subsequently inactivates the enzyme. The equilibrium binding constant, K_I, was determined to be 0.92 ± 0.14 mM, evaluated from direct fitting to the Michaelis–Menten equation using a nonlinear least-squares analysis (Yamaoka et al., 1981). This value is similar to the K_I value of phenylglyoxal determined from steady-state hydrolysis of substrate (0.36 mM at pH 7.0; Zhang & Van Etten, 1990) and indicated a relatively strong binding affinity between BHPTP and EPNP.

The rate of BHPTP inactivation caused by EPNP was strongly reduced in the presence of inorganic phosphate. At pH 7.1 and 27 °C, using 0.704 mM EPNP, the rate constants of inactivation in the absence and presence of 10 mM phosphate were 0.368 and 0.075 h^{-1}, respectively. Similarly, the rate constants of inactivation in the absence and presence of 15 mM phosphate at pH 5.0 and 27 °C, using 1.25 mM EPNP, were 0.0650 and 0.00517 h^{-1}, respectively. If we assume that inorganic phosphate acts to protect the enzyme against inactivation by EPNP by binding to the enzyme active site as a competitive inhibitor, then it should be possible to calculate the phosphate inhibition constant using the expression $K_I = [I]/((k_o/k_i) - 1)$, where K_I is the inhibition constant, [I] is the phosphate concentration, and k_o and k_i are the rate constants for inactivation in the absence and presence of phosphate, respectively. Indeed, at pH 5 and pH 7.1, the values of K_I for inorganic phosphate were 1.3 and 2.6 mM,

Table IV: Concentration Dependence of BHPTP Inactivation by (*R*)- and (*S*)-Benzylglycidol[a]

[(*R*)-benzylglycidol]		[(*S*)-benzylglycidol]	
concn (mM)	k_1 (min^{-1})	concn (mM)	k_1 (min^{-1})
1.0	0.00291	0.5	0.00274
2.0	0.00642	1.0	0.00540
3.0	0.00919	3.0	0.0149
5.0	0.0169	5.0	0.0281
10.0	0.0350	10.0	0.0589
15.0	0.0560	15.0	0.0834
20.0	0.0733		

[a] All measurements were made at 25 °C and pH 7.1, in 50 mM 3,3-dimethylglutarate, 1 mM EDTA, I = 0.15 M buffer.

respectively, effectively identical to those determined by steady-state kinetics (Zhang, 1990). Therefore, it appears that EPNP and inorganic phosphate are competing for the same site. Thus, EPNP is an active site-directed inactivator of BHPTP. The inactivation reaction was irreversible, since extensive dialysis at 4 °C, against pH 7.1 3,3-dimethylglutarate buffer, failed to restore any activity in a sample of EPNP-modified enzyme.

BHPTP was also irreversibly inactivated by (*R*)- and (*S*)-benzylglycidol. The concentration dependencies of inactivation are summarized in Table IV. The data were fitted to $k_1 = ak^n + b$ through nonlinear least-squares analyses (Yamaoka et al., 1981). BHPTP inactivation with (*R*)-benzylglycidol had a kinetic reaction order n of 1.05 ± 0.03 with respect to the reagent, while BHPTP inactivation with (*S*)-benzylglycidol had a kinetic reaction order n of 0.99 ± 0.05 with respect to the reagent. Thus, the reactions of the (*R*) and (*S*) stereoisomers both exhibited a first-order dependence on modifying reagent. Neither of the reagents showed saturation kinetics. The inactivation of BHPTP showed a moderate stereoselectivity with (*R*)- and (*S*)-benzylglycidol, with the (*S*) stereoisomer being 1.7-fold more reactive than the (*R*) stereoisomer.

The pH dependence of the BHPTP inactivation reaction was studied using only (*S*)-benzylglycidol, since the two stereoisomers showed similar kinetic behavior. The modification reaction with 5 mM (*S*)-benzylglycidol had a pH-dependence curve virtually identical to that obtained for the EPNP modification reaction (Figure 7). The curve is consistent with contributions by two ionizable groups with pK_a values of 7.7 ± 0.5 and 9.1 ± 0.9, respectively, as determined from the nonlinear least-squares analysis described above. Again, inorganic phosphate provided strong protection against inactivation, and this was observed with both (*R*)- and (*S*)-benzylglycidol (Figure 9). At pH 7.1 and 25 °C, the first-order inactivation rate constants of BHPTP with 5 mM (*R*)-benzylglycidol in the absence and presence of 10 mM phosphate are 1.69×10^{-2} and 2.06×10^{-3} min^{-1}, respectively, while the first-order inactivation rate constants of BHPTP with 5 mM (*S*)-benzylglycidol in the absence and presence of 10 mM phosphate are 2.81×10^{-2} and 2.48×10^{-3} min^{-1}, respectively.

These and related results for the pH dependencies of the inactivation of BHPTP suggested that iodoacetate, iodoacetamide, EPNP, and (*R*)- and (*S*)-benzylglycidol all modify similar active site functional groups. In order to provide direct structural evidence and to identify these residues, EPNP proved to be ideal. It is an active site-directed inactivator of BHPTP, so that nonspecific modification was minimized. Also, since EPNP has an absorption maximum at 315 nm, it provides a chromophoric label with which to identify labeled peptides after proteolytic digestion of inactivated enzyme.

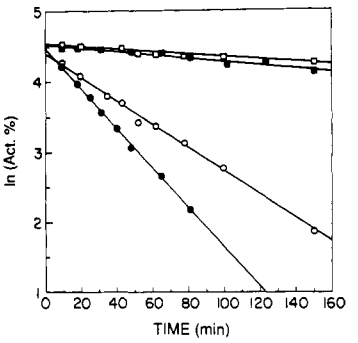


FIGURE 9: Effects of phosphate on the inactivation of BHPTP by (*R*)- and (*S*)-benzylglycidol. The reaction conditions were 25 °C and pH 7.1. Symbols: 5 mM (*R*)-benzylglycidol without phosphate (○) and with 10 mM phosphate (□); 5 mM (*S*)-benzylglycidol without phosphate (●) and with 10 mM phosphate (■).

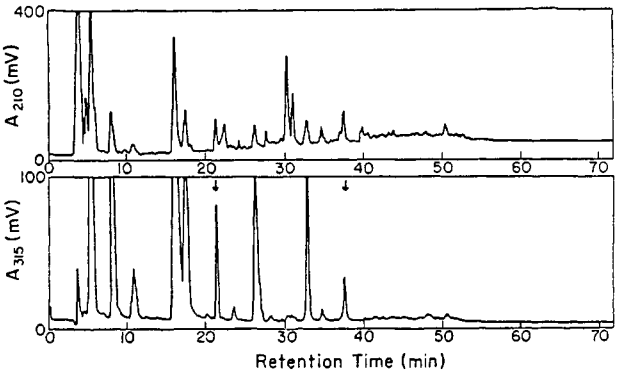


FIGURE 10: Separation of the tryptic peptides by reverse-phase HPLC. The experimental conditions were specified in the text. Peaks no. 22 and no. 38 were peptides that also contained an EPNP label.

A typical reverse-phase HPLC chromatogram of the tryptic digest of EPNP-treated BHPTP is shown in Figure 10. A comparison with the control sample where BHPTP is excluded revealed only two new peaks of absorbance at both 210 and 315 nm. They are peak no. 22, and peak no. 38 (denoted according to their HPLC retention times). The other peaks with both 210- and 315-nm absorbance were not peptides, but they might be due to hydrolyzed and unhydrolyzed TPCK, hydrolyzed and unhydrolyzed EPNP. TPCK did not react with the EPNP-treated BHPTP during the trypsin digestion, since comparison with a control sample did not reveal any extra peaks. Fractions no. 22 and no. 38 were collected and lyophilized, and the peptides were sequenced. Peptide no. 22 corresponded to residues Ala-59 to Arg-64 in the protein sequence (Wo et al., 1992). The initial sequencing yield was 1022 pmol, and the product of cycle no. 4 corresponded to an unknown amino acid derivative with a retention time of 25.34 min, a value that was significantly different from either Leu (24.41 min) or Ile (23.57 min). It was therefore concluded that it was a modified Cys residue (Cys-62) with an EPNP tag on it. Peptide no. 38 corresponded to residues Gln-124 to Arg-147 in the protein sequence. The initial sequencing yield was 323 pmol. The sequencing yield for Arg-147 (cycle no. 24) was 34 pmol. The actual sequencing was done up to cycle no. 26, and no additional sequence was obtained. It is considered that Cys-145 was the only potential nucleophile in this region of the sequence that would give a base stable derivative.

Inactivation by Phenylarsine Oxide. Incubation of enzyme with various concentrations of PAO revealed that the enzyme became inhibited, but in a remarkably slow reaction. Depending on the initial PAO concentration, the time required

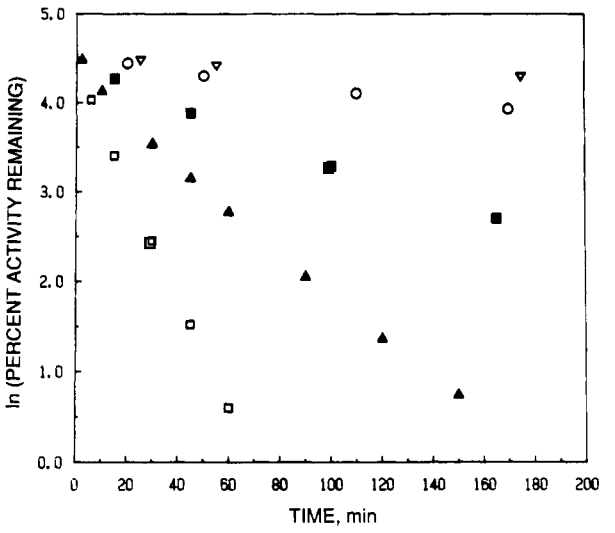


FIGURE 11: Inactivation of BHPTP by phenylarsine oxide at pH 7. The concentrations of PAO were as follows: 6.25 (▽), 12.5 (○), 25 (■), 50 (▲), and 100 μM (□). Pseudo-first-order rate constants calculated from the initial rates of this figure were used to estimate the second-order-rate constant for reaction of BHPTP with PAO.

for nearly complete inactivation ranged from an hour to days (Figure 11). The initial inactivation rates at various PAO concentrations were estimated from Figure 11 and graphed to obtain the second-order rate constant for reaction of PAO and enzyme (the "on" rate). At pH 7, this rate constant k_2 was 8.4×10^2 M^{-1} min^{-1}.

In order to measure the "off" rate, and thus to establish the equilibrium constant for dissociation of the complex (Williams & Morrison, 1979), larger amounts of the inactive enzyme–PAO complex were formed and isolated free of excess PAO. However, even after 12 h, less than 2% recovery of activity could be detected. In contrast, if the complex was incubated with millimolar concentrations of dithiothreitol, the enzyme activity was restored within minutes.

Discussion

The preceding results are consistent with a hypothesis to be developed that there are active site arginine, histidine, and cysteine residues and that ionic interactions exist which alter the reactivity of the cysteine residues.

Arginine Modification. An earlier study involving the attempted inactivation of the human liver enzyme with 2,3-butanedione at 37 °C for 1 h showed no loss of enzyme activity (Taga & Van Etten, 1982), but subsequent studies on the bovine heart and brain enzymes showed inactivation by cyclohexanedione (Zhang & Van Etten, 1990; Saeed et al., 1990). The present chemical modification studies using both phenylglyoxal and 1,2-cyclohexanedione indicate that at least one arginine residue is modified and also that these reagents behave as active site-directed inactivating agents by binding relatively strongly at the active site prior to reaction. 1,2-Cyclohexanedione is a less reactive inactivator than is phenylglyoxal, even though reactions of arginine residues with phenylglyoxal are reported to have a lower (1–2 pH unit) pH optimum than does that of 1,2-cyclohexanedione (Lundblad & Nayes, 1984). For example, at pH 7.0, 27 °C, the phenylglyoxal modification reaction has a second-order rate constant of 80 M^{-1} min^{-1}, while at the same pH at 25 °C the modification by 1,2-cyclohexanedione has a rate constant of only 0.25 M^{-1} min^{-1}. At pH 8.3, 25 °C, the modification reaction involving 1,2-cyclohexanedione has a second-order rate constant of 2.39 M^{-1} min^{-1}. It is an active site-directed inactivator and shows saturation kinetics, with K_i = 12 mM. Although 10 mM phosphate provides good to moderate protection of BHPTP against chemical modification by most reagents including 1,2-cyclohexanedione, it provides only marginal protection against phenylglyoxal modification (see Results). Indeed, at pH 7, phenylglyoxal also acts initially as a competitive inhibitor with a K_i value of 0.36 mM and binds to the enzyme 5-fold more strongly than does phosphate (Zhang & Van Etten, 1990). This is consistent with one of the conclusions drawn from a study of the substrate specificity of BHPTP (Zhang & Van Etten, 1990), namely, that a hydrophobic moiety is required for substrate recognition and binding. Thus, phenylglyoxal has a special affinity to the enzyme, and the irreversible, covalent modification occurs after the binding event. A K_i value of 0.36 mM indicates relatively strong binding, since aside from the potent inhibition exhibited by transition metal oxyanions such as vanadate (Waheed et al., 1988), the only inhibitor with a comparable inhibition constant is arsenate with a K_i = 0.24 mM (Zhang & Van Etten, 1990). Thus, phenylglyoxal is in some ways an active site-directed inactivator of BHPTP. The ineffectiveness of 2,3-butanedione in inactivating the corresponding human liver enzyme (Taga & Van Etten, 1982) may be due to the fact that it lacks a hydrophobic component.

An essential arginine residue has been found in the structurally unrelated phosphatase, human prostatic acid phosphatase (Van Etten et al., 1991). In both types of enzyme, the function of the active site arginine residue is proposed to involve ionic interaction with the phosphate group of the substrates and with the phosphoenzyme intermediate. It may be possible to locate the essential arginine residue on the primary structure of the present enzyme by using ^{14}C-labeled phenylglyoxal (Cheng & Nowak, 1989; Bateman et al., 1989).

Histidine Modification. Chemical modification reactions monitor the ionization states of functional groups in the free enzyme. Results from the present enzyme modification studies with DEP indicate that a histidine residue is present at or near the active site and it has an apparent pK_a of 7.2 ± 0.3. This pK_a value differs markedly from that obtained by a ^1H NMR titration of histidine residues in the bovine liver enzyme (Dayton, 1987). That study revealed only one histidine signal with a pK_a of 8.3. With the exception of one Asn/Asp change which is probably due to a sequencing error by Camici et al. (1989), the bovine liver and bovine heart enzymes have identical amino acid sequences and contain two histidine residues (Wo et al., 1992). The histidine residue with a pK_a of 8.3 that is observed by ^1H NMR titration is different from the active site residue that is identified here by DEP modification, which has a pK_a of 7.2 ± 0.3. It is possible that the active site histidine either has a perturbed relaxation time or that its C-2 proton resonance is markedly shifted by a ring anisotropy effect such that it can not be readily detected in the NMR spectrum. It is anticipated that these possibilities can be tested by using site-directed mutagenesis to construct appropriate histidine mutants. Alternatively, it may be possible to selectively shift the titration curve of the active site histidine residue by the addition of a competitive inhibitor (Jordan et al., 1985).

An active site histidine residue might have two functions. On the one hand, during the phosphorylation of the enzyme, the histidine residue could act as a general acid to donate a proton to the oxygen atom of the leaving group to facilitate the departure of the phenol or alcohol moiety. This is supported by results from a proton inventory experiment, which indicates that a proton is in flight in the transition state of the

phosphorylation event (Zhang & Van Etten, 1991b). On the other hand, in the dephosphorylation of the phosphoenzyme intermediate, the histidine residue could act as a general base to assist the nucleophilic attack by a water molecule. Results from phosphoenzyme trapping experiments (Wo et al., 1992) establish that a cysteine residue is the actual enzymic nucleophile.

Cysteine Modification. The results from cysteine modification reactions at individual pH values are consistent with earlier limited observations that iodoacetate could inactive related enzymes and that phosphate could prevent this inactivation (Lawrence & Van Etten, 1981; Waheed et al., 1988; Camici et al., 1989). However, the present study of the pH dependence of these reactions proved to be quite informative. The pH dependence of the reaction between BHPTP and iodoacetate shows that one cysteine has a pK_a of 6.75 ± 0.34, while the other cysteine has a pK_a of 9.12 ± 0.30. The same experiment with iodoacetamide suggests that one cysteine has a pK_a of 7.52 ± 0.16, while the other has a pK_a of 9.05 ± 0.40. Similar observations have been made on a different enzyme, thioredoxin (Kallis & Holmgren, 1980). Interestingly, in that case too, the pH dependence of the reaction with iodoacetate indicated that one group had a pK_a of 6.7, while the second pK_a was 9.0. For that enzyme, reaction with iodoacetamide showed a similar pH dependence except that the rate of the reaction was approximately 20-fold greater than with iodoacetate at each pH measured. Things are more complicated in the case of BHPTP, where at lower pH (pH < 5.3), iodoacetate reacts with BHPTP more rapidly than does iodoacetamide, while at higher pH, the reverse is true (see Results). The fact that the active site cysteine residue with a pK_a of 6.75 or 7.52 (depending on whether iodoacetate or iodoacetamide is used) shows differential reactivities toward ICH_2COO^- and ICH_2CONH_2 in the range of pH 5–7.5 (see Results) suggests that it interacts electrostatically with a nearby ionizable group, since the major difference between the two reagents is the presence or absence of a negative charge. This is supported by the observation that the reactions of cysteine with these two reagents also show differing sensitivities to D_2O.

At this point, results from the chemical modification studies suggest that at least one arginine residue, two cysteine residues, and one histidine residue are at or near the enzyme active site, while work with the potentially similar brain enzyme also suggests the possibility of an active site lysine (Saeed et al., 1990). The relatively low pK_a values for one of the cysteine residues (6.75–7.52) are consistent with the presence of an arginine residue in the vicinity. However, the abnormal pH dependence of the reaction of BHPTP with a charged reagent like iodoacetate indicates substantial ionic interactions between the active site functional groups, i.e., the ionization of one residue influences the ionization of the other. Charged reagents can be either attracted to or repelled from the vicinity of a functional group in the enzyme active center, thus giving rise to a differential reactivity in enzyme modification. This has been demonstrated by the differences in the comparative rates of modification of the active site cysteine residue by chloroacetamide and chloroacetate in papain (Chaiken & Smith, 1969a,b) and streptococcal proteinase (Gerwin, 1967). Since the active site arginine is always protonated in the pH range studied, the unexpected variation in the reactivity of the cysteine as a function of pH is not due to the presence of the arginine residue. One possibility could be an interaction involving a histidine$^+$/cysteine$^-$ ion pair, similar to that found in papain (Lowe, 1976). Such a possibility is also consistent with a report (Bittencourt & Chaimovich, 1976) that the low molecular weight enzyme from bovine brain contains a sulfhydryl group with high reactivity even at low pH and that its modification by DTNB is very sensitive to increases in the salt concentration. At low ionic strength (20 mM buffer only), the pK_a determined from the k_{inact} versus pH profile is 7.7, while at high ionic strength (20 mM buffer and 300 mM NaCl) a pK_a of 8.4 is determined.

The possibility of an ion pair was tested by studying the D_2O solvent isotope effect on the chemical modification of BHPTP by iodoacetate and iodoacetamide, a charged and a neutral reagent. The reaction of cysteine with ICH_2COO^- exhibits an inverse kinetic isotope effect, while the reaction of cysteine with ICH_2CONH_2 does not. Model system studies show that the alkylation of GSH by ICH_2COO^- or ICH_2CONH_2 exhibits no kinetic solvent isotope effect, consistent with the fact that the model reaction is strictly nucleophilic.

Epoxides react fairly selectively with carboxylate functional groups, especially at low pH values. Tang and co-workers have successfully used a substrate-like epoxide, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), to specifically modify a carboxylate group of the active site Asp residue of pepsin (Tang, 1971; Chen & Tang, 1972; Hartsuck & Tang, 1972). Active site-directed inactivation of lysozyme with an epoxy derivative of a substrate [2′,3′-epoxypropyl β-glycoside of di(*N*-acetyl-D-glucosamine)] led to reaction with the β-carboxyl group of an Asp residue (Eshdat et al., 1974). However, there is also an example of an epoxy derivative of a substrate analogue (glycidol phosphate) that modifies a cysteine residue and causes loss of activity of yeast glyceraldehyde-3-phosphate dehydrogenase (McCaul & Byers, 1976). We explored the action of several epoxide derivatives on the present phosphatase, since we thought it likely that one or more active site nucleophiles might react.

From its structure (Figure 1), it appeared that phosphomycin might possibly act as an active site-directed inactivator of phosphatases, since it has both a phosphonic acid substituent which might aid in binding and an epoxide group which might react with suitably disposed nucleophilic residues at the enzyme active site. However, phosphomycin is neither an active site-directed inactivator nor a covalent irreversible inhibitor. It is merely a reversible inhibitor of BHPTP with a K_i value comparable to that of inorganic phosphate. In contrast, EPNP proved to be an active site-directed inactivator of BHPTP, probably because EPNP possesses an aromatic moiety (Figure 1) which resembles BHPTP substrates (Zhang & Van Etten, 1990). The CH_2O group between the benzene ring and the epoxide may provide the flexibility for optimal interaction between the enzyme and EPNP. This would be consistent with the fact that analogue studies showed that while benzyl phosphate is not a substrate, compounds having two to four methylene groups between the aromatic ring and the phosphate can function as good substrates. Results from arginine modification of BHPTP also emphasize the importance of a hydrophobic binding component.

The inactivation of BHPTP using (*R*)- and (*S*)-benzylglycidol shows moderate stereoselectivity, with the (*S*) stereomer being favored by a factor of 1.7. Enzyme inactivation using (*S*)-benzylglycidol displayed a pH dependence similar to that exhibited by iodoacetate, iodoacetamide, and EPNP, indicating that they react with the same functional residues at the enzyme active site. The second-order rate constants for inactivation using (*S*)-benzylglycidol are similar to those obtained from EPNP modification. Although EPNP is an efficient active site-directed inactivator for BHPTP, it is likely that structural modifications could yield still more efficient

inactivators of this important class of enzyme. Such an affinity label should combine both an aromatic moiety and a phosphate derivative.

The use of EPNP as an affinity label leads to the conclusion that the two cysteine residues located at or near the active site which are modified by iodoacetate, iodoacetamide, EPNP, and (*R*)- and (*S*)-benzylglycidols are Cys-62 and Cys-145. However, these results are not consistent with earlier work using [^{14}C]iodoacetate to modify the bovine liver enzyme. In one case, a small peptide containing Arg and Cys residues was identified (Lawrence & Van Etten, 1981), while in another study, Cys-12 and Cys-17 were identified (Camici et al., 1989). Since both of those experiments were performed in 130 mM cacodylate buffer, which is a moderate competitive inhibitor for the low molecular weight enzyme (Dayton, 1987), those experiments were not performed under optimal conditions.

In the cysteine modification experiments, at least two different cysteine residues are involved (as implied by the two pK_a values determined), yet at each pH value that was measured, from pH 6 to pH 9, using both iodoacetate and iodoacetamide, as 1:1 alkylation stoichiometry is observed. It is likely that the two essential cysteine residues are spatially close to each other and that consequently their chemical modifications are mutually exclusive. There is a good precedent for this in the case of ribonuclease (Crestfield et al., 1963), where iodoacetate reacts with the enzyme with 1:1 stoichiometry at pH 5.5, but in fact two products are obtained, either 1-(carboxymethyl)histidine-119, or 3-(carboxymethyl)-histidine-12. Neither of the modified forms are active, and a doubly-modified enzyme (alkylated at both histidine residues) is not obtained.

PAO is known to be a powerful inactivating agent for enzymes and enzyme complexes that have "spatially close thiols" (Brown et al., 1987). It is an important probe of biochemically significant systems including regulatory pathways. In order to further test the idea that at least two thiols are spatially close in this class of enzyme, we examined the effect of PAO on the enzyme activity. Indeed, low concentrations of PAO slowly but completely inactivated the enzyme (Figure 11).

Although the very slow dissociation rate of the enzyme–PAO complex makes it experimentally difficult to determine a precise equilibrium constant, we may at least make an upper estimate for the numerical value of the dissociation constant. If we make the conservative estimate that no more than 10% of the enzyme activity is restored after 12 h of incubation in dimethylglutarate buffer at pH 7 (in the absence of added DTT or other thiols), then we may use the resulting maximal "off" rate of 1.5×10^{-4} min^{-1} together with the measured "on" rate of 8.4×10^{2} M^{-1} min^{-1}, in order to obtain a maximal estimate for K_{diss} of 1.8×10^{-7} M. Thus, PAO can be classified as a very slow, tight-binding inhibitor (Williams & Morrison, 1979).

One of the two cysteines at the active site of the low molecular weight phosphotyrosyl protein phosphatases may participate in a cysteine-S$^-$ ··· HIm$^+$-histidine ion-pair structure, analogous to that in papain (Polgar & Halasz, 1982; Fersht, 1985; Brocklehurst et al., 1988). This is consistent with the pH-dependence study of the BHPTP-catalyzed hydrolysis of *p*NPP (Zhang, 1990), which displays a constant k_{cat} from pH 4.0 to 7.2. The cysteine residue in the ion-pair structure is proposed to be the actual enzymic nucleophile, and it is proposed to be the one with the lower pK_a (6.75–7.52) for the following reasons: the high enzymic reactivity at acidic pH values implies that the sulfhydryl group of the cysteine residue is ionized, so that the cysteine residue may function as a good nucleophile; the cysteine residue with the lower pK_a is extremely reactive toward ICH_2COO^- even at low pH; and finally, there is a small inverse kinetic solvent isotope effect associated with the inactivation of BHPTP by ICH_2COO^- at pL = 5. The latter two observations fit the proposed model and can be explained as follows. As the ICH_2COO^- molecule approaches the BHPTP active site, the negative charge on the reagent interacts electrostatically with the HIm$^+$ ion of the cysteine/histidine ion pair. This interaction increases the pK_a of the histidine residue while at the same time it decreases the pK_a of the cysteine residue. Indeed, a pK_a of 6.75 was determined from the reaction with ICH_2COO^-, compared to the pK_a of 7.52 obtained from reaction with ICH_2CONH_2. Therefore, at lower pH (pH < 5.3), ICH_2COO^- reacts faster than ICH_2CONH_2, even though ICH_2CONH_2 is generally more reactive than ICH_2COO^-. The observed inverse isotope effect on the rate of alkylation by iodoacetate ($k^H/k^D = 0.757$) can be explained in terms of such an ion-pair model. It could simply originate from an equilibrium solvent deuterium isotope effect on K, estimated to be inverse from the isotopic fractionation factors of the S–H ($\phi \simeq 0.4$) and N–H ($\phi \simeq 1.0$) bonds, so that $K^H/K^D \simeq 0.4$ (Schowen, 1978). An inverse isotope effect has been connected to the disruption of the intimate -S$^-$/-HIm$^+$ ion-pair interaction involving -HIm$^+$ and the reagent (Brocklehurst et al., 1988). Similar observations have been made on papain (Polgar, 1979; Creighton & Schamp, 1980; Wandinger & Creighton, 1980).

The present results thus provide several lines of independent evidence in support of kinetic data that indicate the presence of specific active site residues including nearby thiols in the enzyme (Zhang & Van Etten, 1991a). They also bring attention to several useful probes with which to investigate the biological role of this enzyme and other phosphotyrosyl protein phosphatases that have essential thiols. Finally, although the high molecular weight phosphotyrosyl protein phosphatases including various membrane-bound and receptor-associated forms all have very different primary sequences as compared to the enzyme studied here, it may be useful both to explore with those forms some of the approaches used here and to examine the effects of the active site-directed reagents that have been identified in the present study.

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